OIPE
SEP 21 2006
IAP62
PATENT & TRADEMARK OFFICE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Mark Page et al.	**PATENT**
Serial No.: 10/677,074	Attorney Docket LOR-133.1 (9720/90735)
Filed: October 1, 2003	
For: STABILIZED HBc CHIMER PARTICLES AS THERAPEUTIC VACCINE FOR CHRONIC HEPATITIS	Group Art Unit 1648
Examiner: Zachariah Lucas	

DECLARATION OF DR. GEORGE B. THORNTON

MAIL STOP AMENDMENT
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Sir:

DR. GEORGE B. THORNTON DECLARES:

1. That he is the President of Apovia, Inc. the company that originally owned the subject application;

2. That he is one of the named inventors of the Thornton et al., U.S. Patents No. 4,882,145 and No. 4,818,527 cited in the subject application;

3. That he has read the amended claims that accompany this Declaration;

4. That while Apovia, Inc. owned the invention underlying the subject application, he caused those under his direction and control to carry out a series of studies

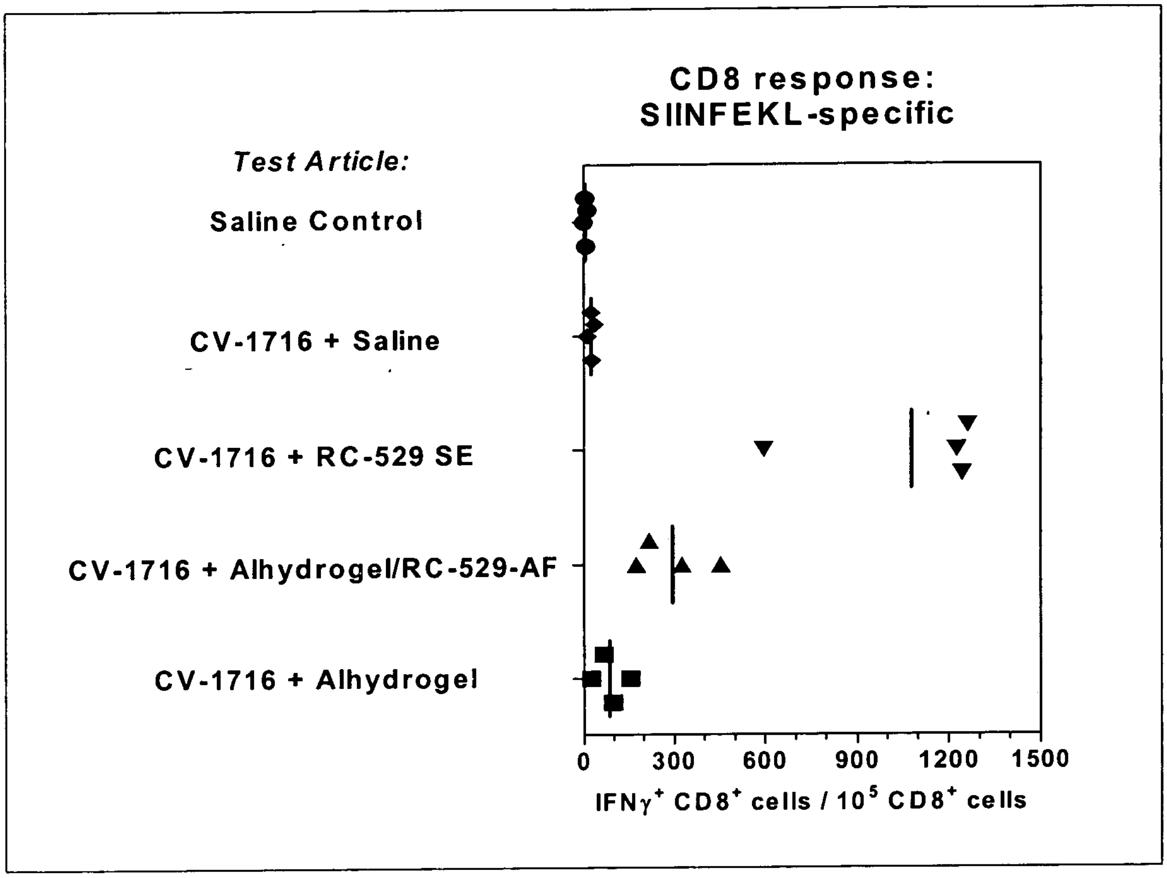
in which various adjuvant compositions were examined for their ability to stimulate T cell responses;

5. That in those studies, different chimer particles were used with different adjuvants in several different studies, as follows:

A. Comparison of Different Adjuvants in Their Capacity to Induce CD8 T Cells

5A1. In order to assess the potential of an HBc chimer to induce antigen-specific CTL, a chimer (designated ICC-1716) carrying the well described SIINFEKL CTL-epitope of ovalbumin between HBc residue 149 and the C-terminal Cys residue was produced and tested in mice. Here, female C57BL/6 mice were used in five groups of four mice each. The mice were injected with 40 μg/animal/injection of immunogen and 10 μg/animal/injection of RC-529 in either the SE (emulsion) or AF (aqueous) formulation, saline, and 100 μg Al^{3+} as Alhydrogel™. Mice were injected once, subcutaneously, and 12 days later the induction of SIINFEKL-specific CD8 T cell responses was assed by IFNγ ELISpot after *in vitro* restimulation of splenocytes with the corresponding CD8-restricted peptide (SIINFEKL).

5A2. As shown below, formulation of CV-1716 emulsified with RC-529-SE induced a 4-fold higher number of IFNγ-secreting CD8 T cells than the Alhydrogel/RC-529-AF formulation. These data provide evidence that a HBc chimer can induce CD8 T cells, and that this capacity is optimal when formulated with an emulsion, and particularly an emulsion containing RC-529-SE as adjuvant rather than the other adjuvants tested.



B. Comparison of Different Adjuvants in Their Capacity to Induce HBc-Specific CD8 (and CD4) T Cell Responses

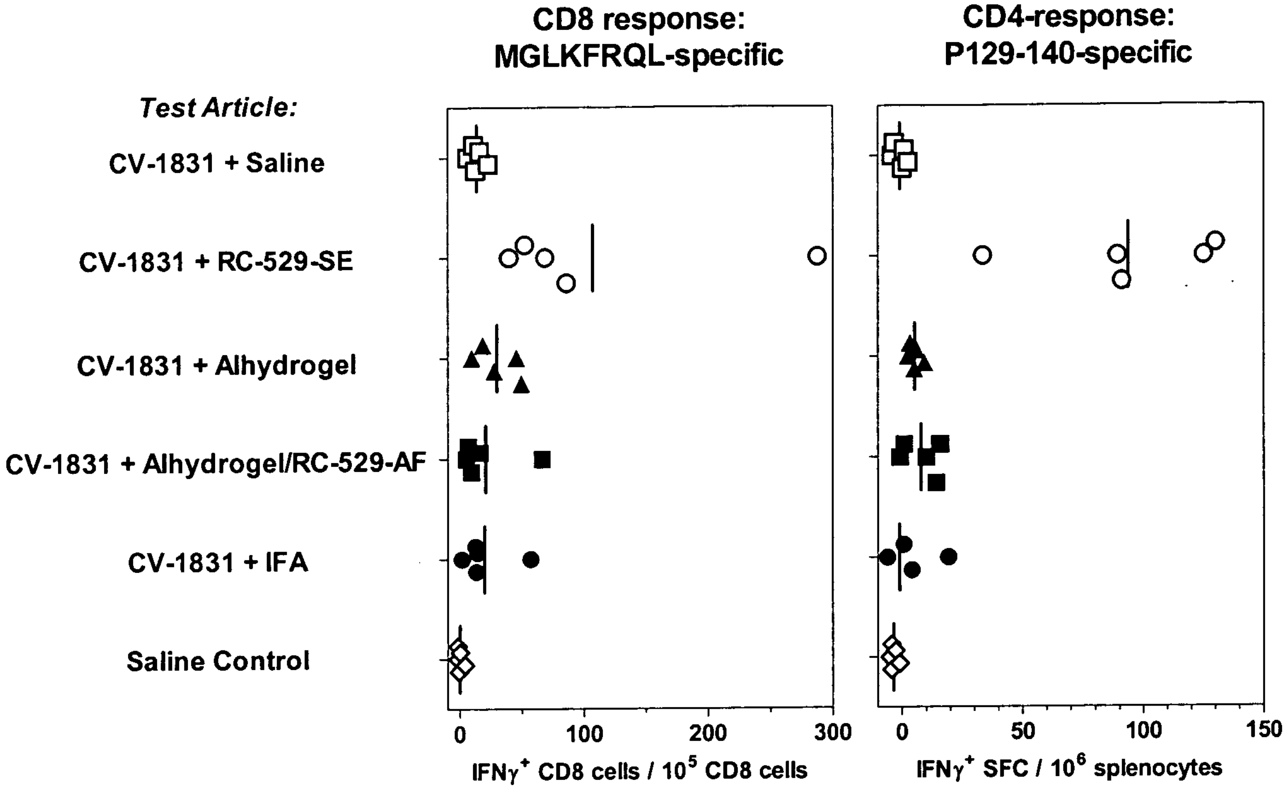
5B1. This study was designed to assess the effect of different adjuvants on the potential of a clinical candidate (CV-1831) to induce HBc-specific IFNγ-secreting CD8 T cells. In addition, the potential of different adjuvants to induce HBc-specific IFNγ-secreting CD4 T cells after a single injection of CV-1831 was assessed.

5B2. CV-1831 particles had a sequence of HBc truncated at amino acid position 149 and stabilized with a C-terminal cysteine. CV-1831 particles also incorporated an additional modification of an alanine residue between loop positions 76 and 77 that reduced HBc-specific antigenicity, and the particles were further stabilized by substituting serine for cysteine at residues 48 and 107, while maintaining the cysteine residue at position 61.

5B3. Mice were injected with 40 μg/animal/injection of antigen and 10 μg/animal/injection of RC-529 in either the SE or AF formulation, and 100 μg Al^{3+}/animal/injection as Alhydrogel™. IFA was added to the CV-1831 solution at a ratio of 1:1. All injections were administered subcutaneously. Ten days after a single injection, HBc-specific CD8 T cell activation was assessed by restimulation of splenocytes with an HBc-specific CD8-restricted peptide (MGLKFRQL; P93-100) and subsequent detection of IFNγ by ELISpot. In addition, HBc-specific CD4 T cell activation was assessed by IFNγ ELISpot after restimulation of splenocytes with the HBc-specific CD4-restricted peptide P129-140.

5B4. Overall, the frequencies of HBc-specific CD8 T cells were low when mice were injected with CV-1831. However, compared to all other adjuvants tested, only the splenocytes from mice injected with CV-1831 formulated in RC-529-SE (the emulsion) revealed some increased frequencies of IFNγ-secreting HBc-specific CD8 T cells as is seen in the left panel of the graphs below.

HBc-Specific CD8 (Left) or CD4 (Right) T Cell Responses of Individual C57BL/6 Mice After a Single Injection of CV-1831 Formulated in Different Adjuvants



5B5. The above right panel shows that increased numbers of HBc-specific IFNγ-secreting CD4 T cells could be detected after restimulation of splenocytes with HBc-specific CD-4-restricted peptide P129-140 only when the mice were once injected with CV-1831 formulated in RC-529-SE, but not when CV-1831 was formulated with another adjuvant.

5B6. In separate studies, the capacity of a CpG-based adjuvant to elicit CD8 T cell responses was assessed and compared with that of RC-529-SE. Although responses were detectable with the CV-1831-CpG formulation, the CD8 T cell response with the RC-529-SE formulation was consistently the strongest.

5B7. In summary, a single immunization with CV-1831 particles in a diluent only resulted in HBc-specific CD4 and CD8 responses when formulated as an emulsion in RC-529-SE, thereby supporting the use of emulsified RC-529-SE as the adjuvant in the proposed human clinical trials. These data provide evidence that, in mice, even a single injection of CV-1831 is able to induce HBc-specific CD4 T cell responses.

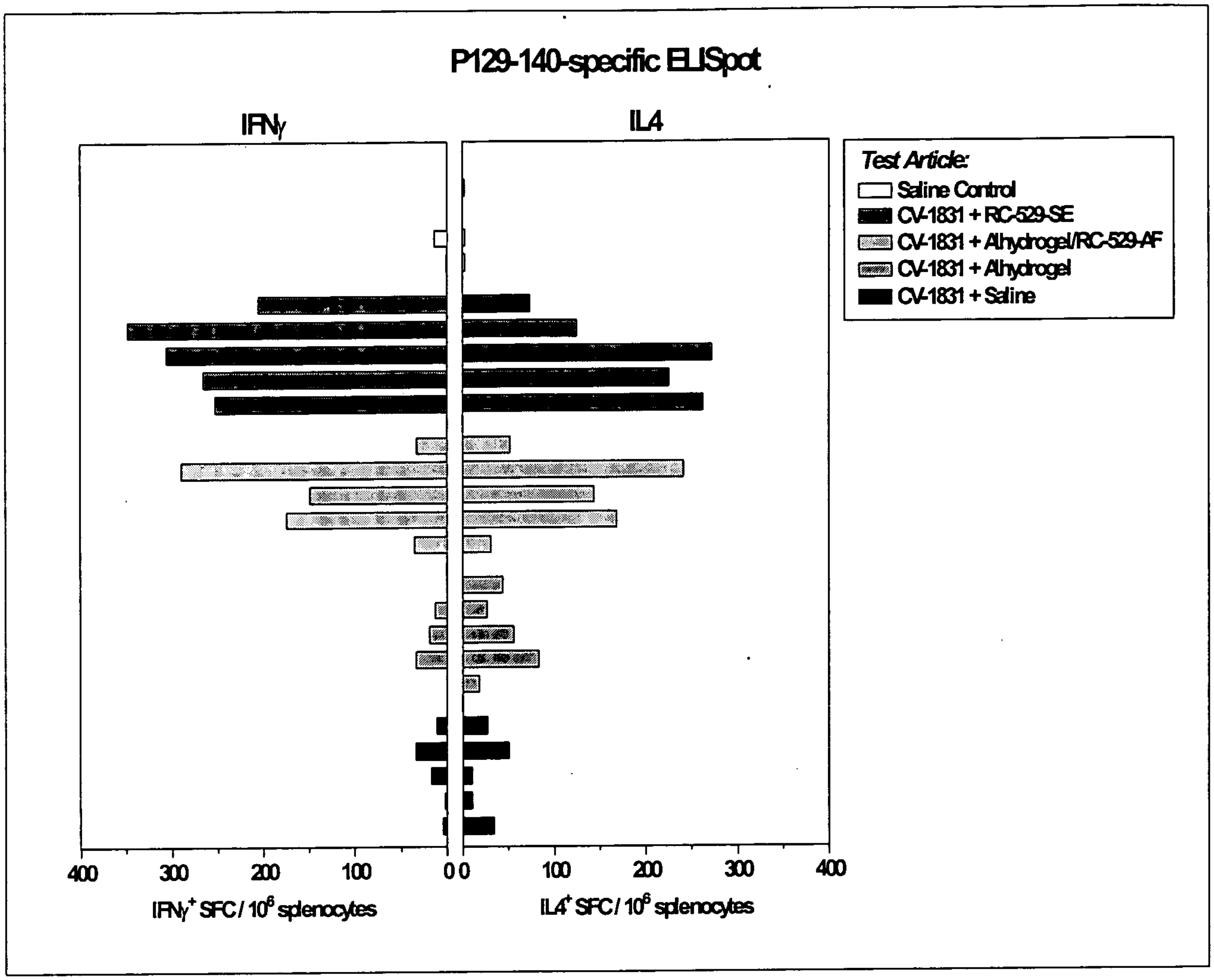
C. Influence of Adjuvant and Boosting Interval on T cell Responses

5C1. The influence of three different adjuvants, RC-529-SE, Alhydrogel™ and Alhydrogel™ + RC-529-AF, on the CD4 T cell response induced by CV-1831 and either 2 or 3 immunizations. C57/6 mice were injected subcutaneously on Days 0, 21, and 42 with 10 μg/animal/injection of antigen, 10 μg of RC-529 in either the SE or AF formulations, and 100 μg Al^{3+}/animal/injection as Alhydrogel™. Cellular immune responses were evaluated by analyzing the cytokine profile (via IL4 and IFNγ ELISpot).

5C2. After two injections of CV-1831 particles adjuvanted with Alhydrogel™ or Alhydrogel™/RC-529-AF, IL4-secreting cells could be detected when splenocytes were restimulated with the HBc-specific CD4-restricted peptide P129-140, indicating a Th2-biased CD4 T cell response. When CV-1831 was adjuvanted with RC-529-SE, both IL4-secreting and relatively high numbers of IFNγ-secreting CD4 T cells were detected, indicating a more Th1-biased HBc-specific immune response compared to the other adjuvants used in this study.

5C3. After three injections (data shown below) of CV-1831 particles formulated in different adjuvants, the Alhydrogel™ formulation again was found to induce moderate numbers of IL4-secreting cells and relatively low numbers of IFNγ-secreting cells when splenocytes were restimulated with the HBc-specific CD4-restricted peptide P129-140, indicating a Th2-biased CD4 T cell response. In contrast to the results after two injections, the splenocytes from mice injected with three doses of CV-1831 particles formulated in Alhydrogel™/RC-529-AF revealed both IL4- and IFNγ-secreting cells, which is indicative of a mixed HBc-specific CD4 T cell response. Mice injected with CV-1831 particles formulated as an emulsion with RC-529-SE exhibited IL4 and slightly higher levels of IFNγ-secreting CD4 T cells, indicating a more Th1-biased HBc-specific immune response compared to the other adjuvants used in this study. Further, detectable numbers of IFNγ-secreting cells could be determined in all five mice when CV-1831 particles were formulated in an emulsion with RC-529-SE, whereas it was detectable in only three of five mice when formulated in Alhydrogel™.

HBc-Specific CD4 T Cell Responses of Individual C57BL/6 Mice After Three Immunizations of CV-1831 Formulated in Different Adjuvants, Given at 3-Week Intervals



D. Influence of Boosting Interval, Vaccine and Adjuvant Dose on T Cell Response

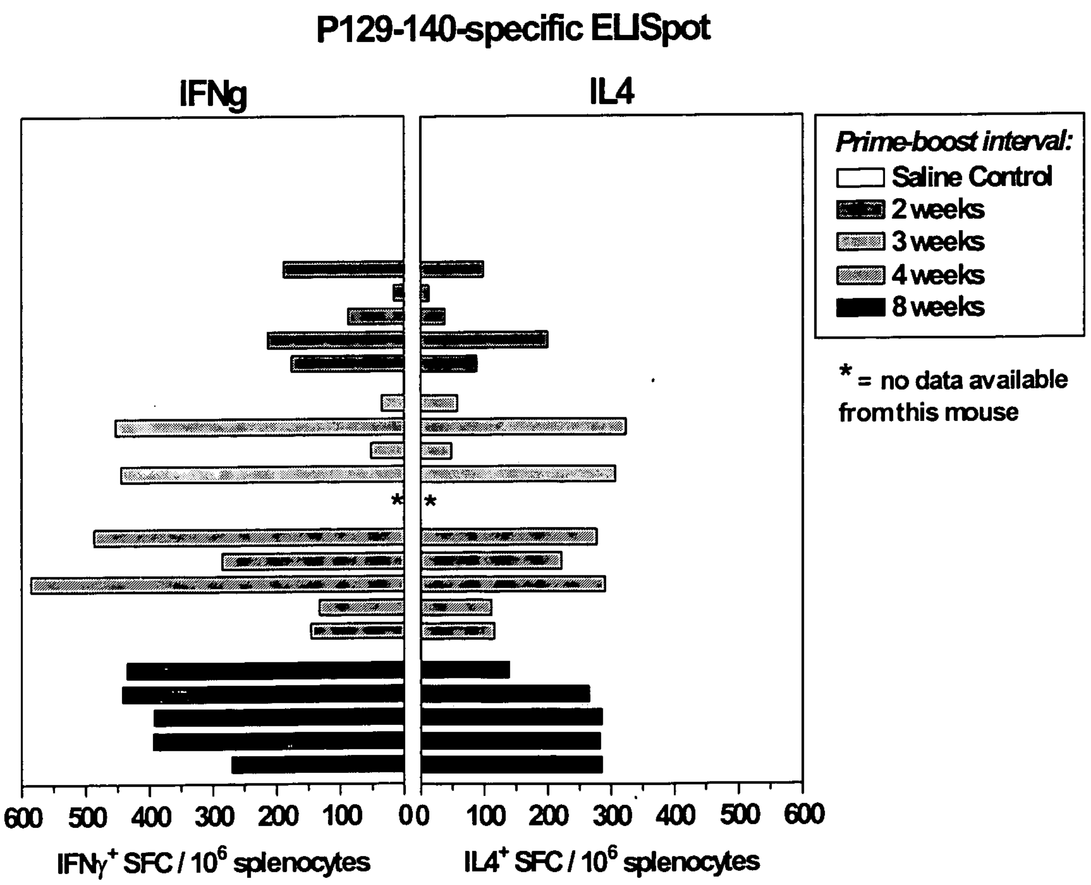
5D1. Two independently conducted studies evaluated the influence of different intervals between the first and second injections of CV-1831 particles plus RC-529-SE on the cellular immune response as well as the dose of vaccine and/or adjuvant.

5D2. C57BL/6 Mice were injected subcutaneously with 10 μg/animal/injection of antigen,

BEST AVAILABLE COPY

10 μg/animal/injection of RC-529 in the SE formulation. Cellular immune responses were evaluated by analyzing the cytokine profile (by quantifying IL4- and IFNγ-secreting cells via ELISpot) 5 days after the second injection. There was no clear difference in the HBc-specific CD4 T cell response between a 3- or 4-week prime-boost interval. However, the response following 2-week interval immunizations seemed to be lower, and the response of the 8-week interval seemed to be less variable. In each group, a mixed CD4 T helper cell response indicated by the release of IFNγ and IL4 was detected. These data are shown in the graph below.

HBc-Specific CD4 T Cell Responses of Individual C57BL/6 Mice After Two Injections of CV-1831 Formulated in RC-529-SE With 2-, 3-, 4-, or 8-Week Intervals

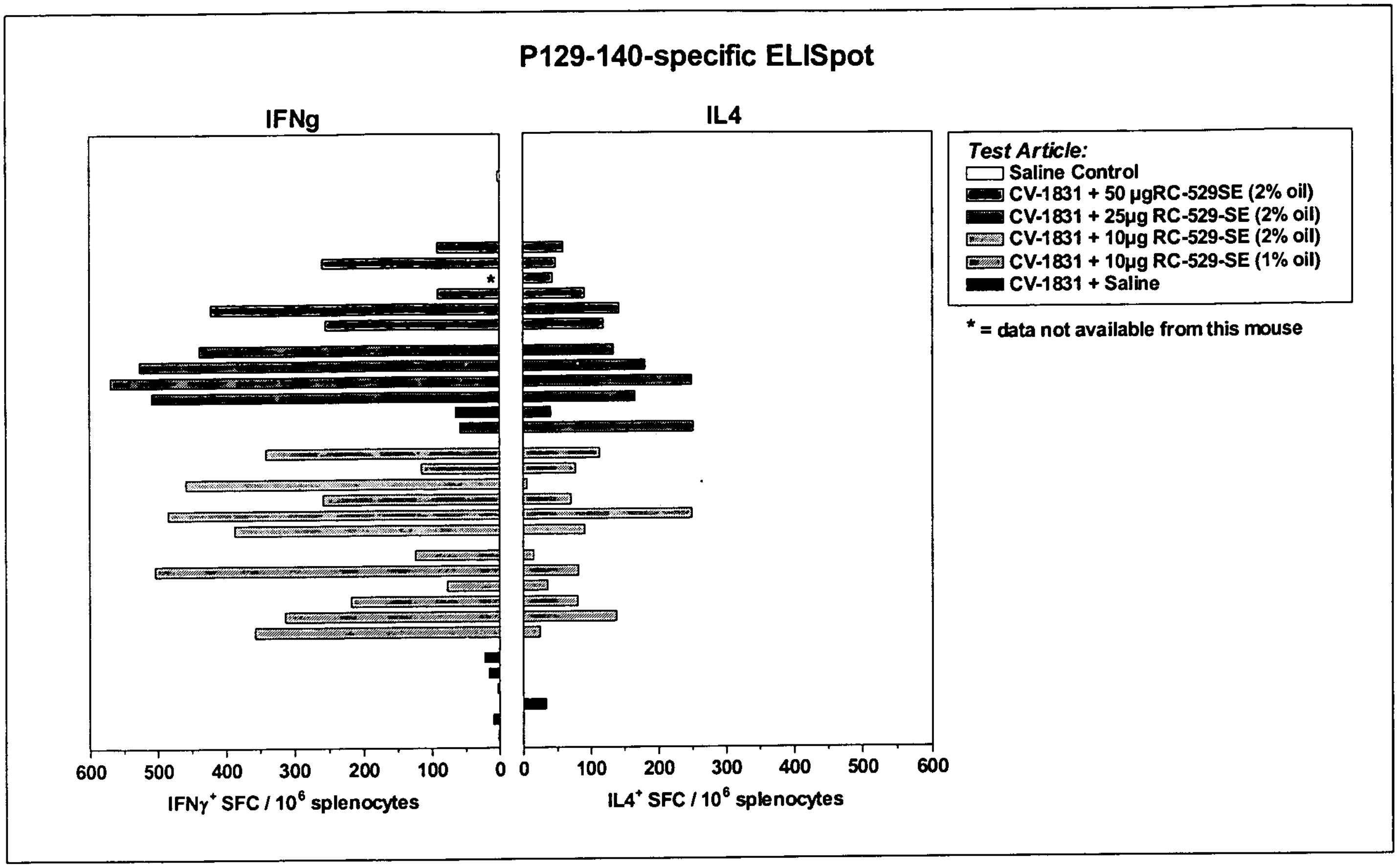


BEST AVAILABLE COPY

5D3. For the assessment of dose-response effects, C57BL/6 mice were injected, subcutaneously, with 10 μg/animal/injection of antigen on Day 0 and Day 29. Cellular immune responses were evaluated by analyzing the cytokine profile (via IL4 and IFNγ ELISpot) of isolated and *in vitro* restimulated splenocytes 5 days after the second injection.

5D4. The graph below shows that cytokine-release after restimulation of splenocytes with the HBc-specific CD4-restricted peptide P129-140 was only detectable when CV-1831 was formulated in an emulsion with RC-529-SE as adjuvant, but almost undetectable when formulated in saline. No substantial difference in the frequency of cytokine-secreting cells was observed between the groups that received 10 μg and 25 μg RC-529. A decrease in the frequency of cytokine-secreting cells was noted in the group that received 50 μg RC-529. The reasons for this observation remain to be elucidated. With respect to the final oil concentration at the injection site there was no substantial difference seen between the groups that received 1% oil and 2% oil. Splenocytes from mice of both groups showed high frequencies of IFNγ-secreting cells and lower frequencies of IL4-secreting cells.

HBc-Specific CD4 T Cell Responses of Individual C57BL/6 Mice After Two Injections of CV-1831 Formulated in Different Doses of RC-529-SE



E. Clinical Experience With a Related HBc Chimer vaccine

5E1. Malaria results from infection with the parasite *Plasmodium falciparum*. ICC-1132 is a particle disclosed in US Patent No. 6,942,866, a patent owned by Apovia, Inc. That particle is similar to the CV-1831 particle. However, ICC-1132 particles contain a circumsporozoite (CS) epitope [NANPNVDP(NANP)$_3$] inserted into the immunodominant region of the HBc protein between

BEST AVAILABLE COPY

residues 78 and 79 as the one to about 40 amino acid residues containing one or more chemically non-reactive heterologous amino acid residues that render the immunogenic particles less antigenic than the native HBc particles, as compared to the single alanine of ICC-1831, and includes a *Plasmodium falciparum* T cell epitope [GIEYLNKIQNSLSTEWSPCSVT] bonded to HBc residue position 149 that provides a C-terminal cysteine residue.

5E2. ICC-1132 particles have been evaluated clinically in four Phase 1 studies in Europe (two in the United Kingdom and one in Germany) and the US, and one Phase 2a (challenge) study in the UK. All of the ICC-1132 particle studies have been conducted in healthy volunteers. Given the focus on cellular responses here, only the cellular responses to the ICC-1132 will be discussed.

5E3. In a Phase 1 study conducted in Cardiff, Wales, a cultured ELISpot assay was used to assess cellular immune responses in vaccinees receiving ICC-1132 particles formulated on alum in saline. This assay identified ICC-1132- and CS-specific IFN-γ-secreting T cells in 50% of the vaccinees that received the 50 μg dose of alum-adsorbed vaccine. The results of this study were published, in part, in Nardin et al., 2004 *Infect. Immun.* **72(11)**:6519-6527) that is attached to this declaration.

5E4. In a study conducted in Tübingen with ICC-1132 particles formulated with Montanide™ ISA 720 as adjuvant in a water-in-oil emulsion, a combination of *in vitro* proliferation with cytokine secretion, T cell culture and intracellular cytokine staining was used to evaluate the cellular responses. PBMCs of the majority of volunteers (6/8) who received a single 20 μg dose of vaccine showed pronounced proliferation after *in vitro* restimulation with CS protein (6/8).

5E5. Consistent with the polarized Th1-type IgG1 and IgG3 antibody responses observed, supernatants of proliferating PBMC (following stimulation with ICC-1132 or HBc) contained significant amounts of IFN-γ. Incubation with rCS also elicited IFN-γ production, indicating parasite-specific Th-1 cells were induced by immunization with ICC-1132/ISA 720. These data were published, in part, in Oliveira et al., 2005 *Infect. Immun.* **73(6)**:3587-3597 that is attached to this declaration.

5E6. Intracellular staining identified both $CD4^+$ and $CD8^+$ T cells as the sources of this cytokine, most notably in response to stimulation with a CS-specific 20-mer peptide (unpublished data). Outstanding in this regard was the fact that the numbers of CS-specific IFN-γ-positive $CD4^+$ T cells were equivalent to or even greater than those observable after 3 immunizations with a similar vaccine (similar CS epitopes) based on the hepatitis B surface antigen [Lalvani *et al*, 1999 *J. Infect. Dis.* **180**(5):1656-1664].

5E7. Short-term T cell lines established from the 20 μg vaccinee group confirmed the capacity for CS-specific T cell proliferation and cytokine (IL-2, IFN-γ) secretion. Overall in this study, T cell-associated ICC-1132- or parasite antigen-specific IFN-γ activity, respectively, was detectable in 13/16 and 12/16 of vaccinees that received either 20 or 50 μg of vaccine. A majority (9/16) also responded to HBc particles.

5E8. These studies demonstrated for the first time that ICC-1132 particles elicit appropriately targeted epitope-specific responses from both $CD4^+$ and $CD8^+$ T cells in humans. Perhaps of equal importance is the fact that these responses were detectable at appreciable levels after only a single immunization. No significant safety concerns have been observed in any of these studies.

6. That the claims recite the presence of an insert in the HBc immunodominant loop, in which the insert has a length of one to about 40 amino acid residues and contains one or more chemically non-reactive heterologous amino acid residues that render the immunogenic particles less antigenic than the native HBc particles. Apovia scientists illustrated that lessening of antigenicity with several chimer constructs that are useful in a claimed method.

A. Antigenicity of Malaria-containing Chimers

6A1. A compilation of antigenicity data from Tables 13A-C of Apovia's US Patent No. 6,942,866 is shown in the table below. Immunogenicities as end point dilution values of monoclonal antibodies directed to the HBc immunogenic loop (MoAB-3120 and MoAB-3105) against HBcAg itself and various chimeer constructs containing a malarial peptide in the immunogenic loop are illustrated. Parenthesized values are from a separate study with MoAB-3120 assayed against chimers containing the same malarial peptide insert placed between residues 77 and 78 (V1.Pf1) or between residues 78 and 79 (V2.Pf1). The peptide inserts are shown using single leteter amino acid residue code.

Compiled Tables 13 of US Patent No. 6,942,866

Name	*HBc-inserted* *P.falciparum* B Cell Epitope	Antigenicity End Point Dilution	
		MoAB-3120 (Separate Study)	MoAb-3105
HBcAg	None	625,000 (409,600)	1,300,000
V1.Pf1	$(NANP)_4$	(102,400)	Zero
V2.Pf1	$(NANP)_4$	(400)	Zero
V12.Pf1	$(NANP)_4$	-	Zero
V12.Pf3	NANPNVDP$(NANP)_3$NVDP	80,000	Zero
V12.Pf3.1	NANPNVDP$(NANP)_3$	20,000	-
V12.Pf3.2	$(NANP)_3$NVDPNANP	10,000	-
V12.Pf3.3	NANPNVDP$(NANP)_3$-NVDPNANP	10,000	-
V12.PF3.4	NPNVDP$(NANP)_3$NV	80,000	-
V12.PF3.5	NPNVDP$(NANP)_3$NVDP	40,000	-
V12.PF3.6	NPNVDP$(NANP)_3$-NVDPNA	80,000	-
V12.PF3.8	NVDP$(NANP)_3$NVDP	80,000	-
V12.PF3.9	NVDP$(NANP)_3$NVDPNA	160,000	-
V12.PF3.10	DP$(NANP)_3$NV	10,000	-

V12.PF3.11	DP(NANP)$_3$NVDP	80,000	-
V12.PF3.12	DP(NANP)$_3$NVDPNA	80,000	-

B. Antigenicity of Influenza-containing Chimers

6B1. A compilation of antigenicity data from Example 11 of published US 20060115489 (WO/2005/055957) owned jointly by Apovia and Vlaams Interuniversitaie Instituut Voor Biotechnologie (Belgium) is shown in the table below. Monoclonal antibody 14C2 immunoreacts with the M2e peptide of influenza. Monoclonal antibody 3105 is as above and reacts with the immunogenic loop of HBc. Particles CV-1123 are C-truncated HBc at position 149 with a cysteine at position 150 and no insert into the immunodominant loop. Particles CV-1559 contain full length HBc plus N-terminal M2e peptide of influenza [Neirynck et al., (October 1999) *Nature Med.*, **5(10)**:1157-1163]. Particles CV-1604 are C-truncated HBc at position 149 with a cysteine at position 150, with M2e peptide of influenza at the N-terminus. Particles CV-1569 are C-truncated HBc at position 149 with a cysteine at position 150, and with an M2e peptide inserted in the loop between residues 78 and 79 or are like CV-1123 particles with an M2e peptide inserted into the loop between residues 78 and 79.

Example 11, US 20060115489 (WO/2005/055957)

Particle	Monoclonal Antibody	
	14C2	3105
CV-1123	-	+
IM2HBc/ CV-1559	+	+
CV-1604	+	+
CV-1569	+	-

"+" = immunoreaction. "-" = no immunoreaction.

6B2. As is seen from the above results, only the particles containing the insert into the loop did not react with the anti-loop monoclonal antibodies, MoAB-3105.

7. That as noted in Paragraphs 5B1 and 5B2, clinical candidate CV-1831 particles had a sequence of HBc truncated at amino acid position 149 and that was stabilized with a C-terminal cysteine. CV-1831 particles also incorporated an additional modification of an alanine residue between loop positions 76 and 77 that reduced HBc-specific antigenicity, and the particles were further stabilized by substituting serine for cysteine at residues 48 and 107, while maintaining the cysteine residue at position 61.

8. That a comparison of a pair of C-truncated particles (149+C), that each contain an alanine insertion between amino acids 76 and 77, under boiling conditions illustrated the unexpected added stability of the CV-1831 particle structure as compared to particles of a similar

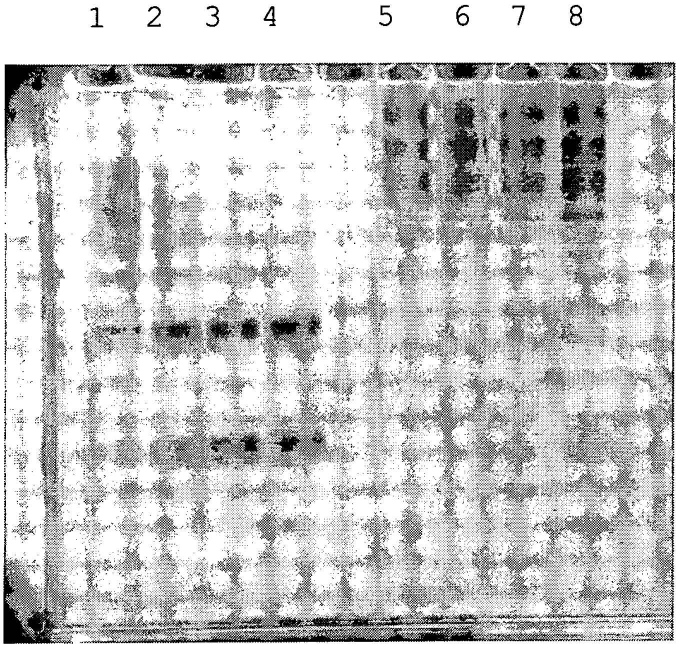
structure that contained the native cysteines at residues 48 and 107 and the inserted alanine between residues 76 and 77 (CV-1809).

9. That one set of particles used in this study were designated CV-1812 and had the identical structure to the before-described CV-1831 particles, but were expressed using a plasmid conferring ampicillin (CV-1812) as compared to kanamycin (CV-1831) resistance. The other 149+C particles were a non-mutated C48, C107 version designated CV-1809 particles.

10 That the study results indicated that the particles with the cysteine-to-serine mutations at HBc positions 48 and 107 (C48S, C107S; CV-1812), while maintaining the cysteine at position 61, displayed greater thermal stability under non-reducing conditions, as determined by retention of higher order structure following boiling, as opposed to the non-mutated CV-1809 particles that exhibited substantial quantities of monomers and dimers, in addition to higher order structures.

11. That these results are shown below in the figure wherein barb-headed arrows indicate dimers, and the flat-headed arrows show monomers. Lanes 1-4, CV-1809; Lanes 5-8, CV-1812. Lanes 1 & 5, no heat; Lanes 2 & 6, 50°C 10 minutes; Lanes 3 & 7, 70°C 10 minutes; Lanes 4 & 8, boiled 5 minutes.

Non-reducing SDS-PAGE gel analysis of CV-1809 and CV-1812



BEST AVAILABLE COPY

12. That all statements made herein of his knowledge are true and all statements made on information and belief are believed to be true; and further, these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code, and that such willful, false statements may jeopardize the validity of the above-identified application or any patent issuing thereon.

15 SEPT 2006

George B. Thornton

Date

CERTIFICATE OF MAILING

I hereby certify that this Declaration, as well as the Amendment and Reply and its stated enclosures are being deposited with the United States Postal Service with sufficient postage as First Class Mail in an envelope addressed to: Mail Stop AF Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on September 15, 2006.

By Edward P. Gamson

Edward P. Gamson